IN THE DRAWINGS:

Please delete originally filed Figure 3.

REMARKS

Applicants have deleted Figure 3 from the specification. This figure was submitted as an informal drawing with the application. Figures 4 – 8 have been renumbered as Figures 3 – 7. Applicants have determined that originally filed Figure 3 is not necessary for understanding the present invention. In addition, this Figure was deleted from the parent case (U.S. Patent Appln. Ser. No. 08/952,445, filed 11/18/97), in an Amendment mailed August 2, 2000. No new matter has been added to the application by this deletion.

Attached hereto is a marked-up version of the changes made to the specification by the current amendment. The attached pages are captioned **"Version with Markings to Show Changes Made."**

If in the opinion of the Examiner a telephone conference would expedite the prosecution of the subject application, the Examiner is encouraged to call the undersigned at (650) 846-5838.

Respectfully submitted,

Date: 3/13/02

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Version with Markings to Show Changes Made

The present invention provides for a purified 38kD esterase which is derived from *Aspergillus niger.* Further, a DNA sequence coding for the 38kD esterase comprising a DNA as shown in Figs. [5] 4A-4E (SEQ. ID NO: 27); a DNA which encodes the amino acid sequence also shown in Figs. [5] 4A-4E (SEQ. ID NO: 28); a DNA which encodes an esterase which comprises an amino acid segment which differs from the sequence in Figs. [5] 4A-4E, provided that the DNA encodes a derivative of the 38kD esterase specifically described herein; and a DNA which encodes an esterase that comprises an amino acid segment which differs from the sequence in Figs. [5] 4A-4E, provided that the DNA hybridizes under low-stringency conditions and/or standard stringency conditions, as defined below, with a DNA comprising all or part of the DNA in Figs. [5] 4A-4E are provided. The present invention further encompasses vectors which include the DNA sequences described above, host cells which have been transformed with such DNA or vectors, fermentation broths comprising such host cells and esterase proteins encoded by such DNA which are expressed by the host cells. Preferably, the DNA of the invention is in substantially purified form and is used to prepare a transformed host cell capable of producing the encoded protein product thereof. Additionally, polypeptides which are the expression product of the DNA sequences described above are within the scope of the present invention.

Figs. 2A-2B illustrate[s] the DNA sequence (SEQ. ID NO:25) with deduced introns and amino acid sequence (SEQ. ID NO:26) of a 650 base pair fragment corresponding to the gene encoding a 38kD esterase isolated from *Aspergillus niger.*

[Fig. 3 illustrates the band corresponding to the 38kD esterase after purification.]

Fig. [4] 3 illustrates a restriction map of a DNA fragment containing the gene encoding the 38kd esterase.

Figs. [5] 4A-4E illustrate[s] the complete DNA (SEQ. ID NO:27), with highlighting to point out the signal sequence, intron and various restriction endonuclease sites, and amino acid sequence (SEQ. ID. NO:28) corresponding to the gene encoding the 38 kD esterase isolated from *Aspergillus niger.*

Fig. [6] 5 illustrates the DNA sequence of the gene encoding the 38 kD esterase (SEQ. ID. NO:29).

Fig. [7] 6 illustrates a southern blot gel showing hybridization between a DNA probe derived from the 38 kD esterase of the invention and several other filamentous fungi ("gel 1").

Fig. [8] 7 illustrates a southern blot gel showing hybridization between a DNA probe derived from the 38 kD esterase of the invention and several other filamentous fungi ("gel 2").

DETAILED DESCRIPTION OF THE INVENTION

"Esterase" or "esterolytic activity" means a protein or peptide which exhibits esterolytic activity, for example, those enzymes having catalytic activity as defined in enzyme classification EC 3.1.1. Esterolytic activity may be shown by the ability of an enzyme or peptide to cleave ester linkages, for example, feruloyl, coumaroyl or acetyl xylan groups, from organic compounds in which they are known to exist, e.g., primary and secondary cell walls. Preferably, the esterase comprises an esterolytic activity which cleaves the ester linkage of phenolic esters such as: [5-*O*-((*E*)-ferloyl)-α -L-arabinofuranosyl] (1→3)-O-β-D-xylopyranosyl-(1→4)-D-xylopyranose (also known as FAXX); [5-*O*-((*E*)-feruloyl)-α -L-arabinofuranosyl] (1→3)-O-β-D-xylopyranose (also known as FAX); *O*-β-D-xylopyranosyl-(1→4)-*O*-[5-*O*-((*E*)-feruloyl)-α- arabinofuranosyl-(1→3)]-*O*-β-D-xylopyranosyl-(1→4)-D-xylopyranose (also known as FAXXX); [5-*O*-((*E*)-*p*-coumaroyl)-α -L-arabinofuranosyl] (1→3)-O-β-D-xylopyranosyl-(1→4)-D-xylopyranose (also known as PAXX); [5-*O*-((*E*)-*p*-coumaroyl)-α -L-arabinofuranosyl] (1→3)-O-β-D-xylopyranose (also known as PAX); *O*-β-D-xylopyranosyl-(1→4)-*O*-[5-*O*-((*E*)-*p*-coumaroyl)-α- arabinofuranosyl-(1→3)]-*O*-β-D-xylopyranosyl-(1→4)-D-xylopyranose (also known as PAXXX) and other ester linked phenolic oligosaccharides as are known in the art. Such esterases are generally referred to as ferulic acid esterase (FAE) or enzymes having feruloyl esterase activity. It has surprisingly been discovered that an esterase having ferulic acid esterase activity which may be purified from *Aspergillus niger*, as described herein, and having an amino acid sequence as shown in Figs. [5] 4A-4E, further has activity on sugar beet pulp and also proteolytic and lipolytic activity. Thus, according to a particularly preferred embodiment of the present invention, an esterase and/or a DNA encoding that esterase is provided which esterase also has lipolytic and/or proteolytic activity. Accordingly, the esterase of the invention having

Thus, according to a particularly preferred embodiment of the present invention, an esterase and/or a DNA encoding that esterase is provided which esterase also has lipolytic and/or proteolytic activity. Accordingly, the esterase of the invention having measurably significant esterolytic activity on feruloyl and coumaroyl esters also has proteolytic and lipolytic activity.

The DNA probe derived from the sequence in Figs. [5] 4A-4E or [6] 5 should be isolated by electrophoresis in 1% agarose, the fragment excised from the gel and recovered from the excised agarose. This purified fragment of DNA is then random prime ^{32}P labeled (using, for example, the *Megaprime* labeling system according to the instructions of the manufacturer (Amersham International plc, Buckinghamshire, England)). The labeled probe is denatured by heating to 95° C for 5 minutes and immediately added to the prehybridization solution above containing the membrane. The hybridization reaction should proceed for an appropriate time and under appropriate conditions, for example, for 18 hours at 37°C with gentle shaking. The membrane is rinsed (for example, in 2X SSC/0.3% SDS) and then washed with an appropriate wash solution and with gentle agitation. The stringency desired will be a reflection of the conditions under which the membrane (filter) is washed.

Figs. [5] 4A-4E and [6] 5 illustrate[s] the amino acid sequence and DNA sequence of a novel esterase derived from *Aspergillus niger.* The isolated esterase has a molecular weight of about 38kD (as shown on SDS-PAGE), a pI of about 2.8 (as shown on IEF), a pH optimum of about 5.1 on methyl ferulate, a temperature optimum of about 55°C and activity on coumaroyl and feruloyl esters, and sugar beet pulp. The FAE gene shown in Figs. [5] 4A-4E (SEQ. ID NO: 27) is approximately 2436 base pairs in length including deduced intron sequence and, if expressed, will encode the herein identified esterase from *Aspergillus niger* (hereinafter the "38kD esterase"). For the purposes of the present invention, the term "38kD esterase" means an esterase derived from *Aspergillus niger* corresponding to the esterase specifically exemplified herein. The DNA provided in Figs. [5] 4A-4E or [6] 5 will be useful for obtaining homologous fragments of DNA from other species, and particularly from anaerobic fungi, which encodes an enzyme having esterolytic activity.

The inventors herein hypothesized that a specific property of the protein or the purified protein composition was preventing obtaining purified representative protein. To test this theory, the product protein from above was analyzed via iso[l]electric focusing gel at pH 2-4 under various conditions. [As shown in Fig. 3] [p] Protein samples taken from purification steps along the purification method described above appeared to be a single band of highly purified protein. A second analysis was performed in which the purified protein was subjected to denaturing conditions of an SDS-PAGE and the results western blotted. As shown in Fig. 1, the resultant protein showed a number of bands indicating either some degeneration of the protein or other compounds hidden during the IEF gel. Sequencing of each of the numerous bands showed that each possessed an identical N-terminal sequence and that proteolysis appeared to be occurring from the carboxy terminal.

A restriction digest was made on genomic DNA prepared as in Example 3. The reaction included 50 μl of genomic DNA, 50 μl of 10X restriction endonuclease buffer H (Boehringer Mannheim), 25 μl of EcoRI (10 units/μl, Boehringer Mannheim), 375 μl of distilled water. The reaction proceeded at 37°C for 6 hours. The digestion mixture was electrophoresed through 0.8% agarose. Fragments between a range of approximately 5 kb to 6 kb were cut from the gel in three approximately equal slices. The three pools of DNA fragments contained within the three gel slices each possessed a slightly different range of fragment lengths. The DNA was recovered from the slices of agarose using *QIAquick Gel Extraction* columns, following the instructions of the manufacturer (Qiagen, Inc., Chatsworth, CA). Approximately 1/10 of each pool of recovered DNA was electrophoresed in 0.8% agarose and southern hybridized to the 650 base pair fragment as described above. The pool of DNA which gave the strongest hybridization signal was ligated into an EcoR I digested *E.coli* vector (for example *pLITMUS 28*, New England Biolabs), which was then transformed into *E.coli*. The *E.coli* transformants were plated out on 5 plates at a concentration of approximately 500 colonies per plate (150 mm diameter plate). Colony lifts were performed on the plates using Maximum Strength Nytran Plus membranes. A southern hybridization was performed using the 650 base pair fragment. Four strong hybridization signals were obtained. Colonies putatively corresponding to the four

hybridization was performed using the 650 base pair fragment. Four strong hybridization signals were obtained. Colonies putatively corresponding to the four strong hybridization signals were grown up and their plasmid DNA recovered. Restriction digests on the plasmid DNA were made using restriction enzymes that were chosen based on sites within the 650 base pair fragment. One plasmid restriction digest gave restriction fragments consistent with the known restriction sites within the 650 base pair fragment. Upon DNA sequencing, this clone was revealed to contain the 650 base pair sequence that was obtained through PCR described in example 2. Restriction mapping of this clone reveals the 650 base pair fragment to lie within the approximately 5.5 kb of cloned genomic DNA sequence. Based on this procedure, DNA encoding the entire gene of the 38 kD esterase was isolated corresponding to the sequence provided in Fig. [6] 5 (SEQ. ID. NO:27) encoding a protein having the amino acid sequence of Fig. [6] 5 (SEQ. ID. NO:28).

The genomic DNA was digested with two restriction enzymes, Bgl II and Ppu10 I, and then electrophoresed through 0.7% agarose in two different gels. Genomic DNA fragment sizes separated on the agarose gel ranged from about 1kb to about 20 kb. The gels were depurinated and denatured and Southern blotted onto Nytran plus. The membranes were air dried and hybridized with the 650 base pair fragment ^{32}P labeled. The membranes were washed under low stringency conditions, followed by washing under standard stringency conditions. The membranes were then autoradiographed. The reproduced gels are provided in Figures [7] 6 and [8] 7.